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# Stability and dynamics of enzyme activity patterns in the rice rhizosphere: Effects of plant growth and temperature

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## ABSTRACT

The rhizosphere is the most dynamic hotspot of microbial activity in the soil. Despite these dynamics, the spatial pattern of many rhizosphere properties may remain stable because they are continuously reproduced in the changing environment. Low substrate concentration can strongly reduce the rate response of an enzymatic reaction subjected to increased temperature and is recognized as a canceling effect on enzyme temperature sensitivity. Carbon input from rhizodeposits affects C availability in the rhizosphere, and thus the enzyme activities responsible for organic matter decomposition, and their temperature sensitivities, upset the dynamics and stability of biochemical processes in the rhizosphere. However, it is unclear whether a canceling effect occurs in the rhizosphere. We studied temperature effects on chitinase and phosphatase during rice (*Oryza sativa* L.) growth at 18 and 25 °C. The spatial distribution of enzyme activities was imaged using soil zymography and showed that the overall activities of these enzymes increased with temperature but decreased with rice growth. The temporal dynamics of hotspot areas were enzyme-specific. During growing days 14–30, hotspot areas decreased from 2–2.5% to 0.3–0.5% for chitinase, but increased from 2% to 6–7% for phosphatase. The distribution pattern of both enzymes shifted from being dispersed throughout the soil to being associated with the roots. For the first time, we showed the extent of rhizosphere enzyme activity in paddy soil and demonstrated that it is temporally stationary and independent of temperature. However, the temperature sensitivity of enzyme activities declined radically ( $Q_{10} \sim 1.3$ – $1.4$ ) at the root surface compared to that of bulk soil ( $Q_{10} \sim 1$ ). We conclude that the spatio-temporal pattern of rhizosphere enzymatic hotspots is mainly affected by plant growth. High temperature sensitivity ( $Q_{10} > 1$ ) at the root-soil interface for the tested enzymes revealed that warming will lead to faster nutrient mobilization in the rhizosphere than in root-free soil.

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## 1. Introduction

The rhizosphere—the volume of soil affected by plant roots—is one of the most dynamic spheres in the biosphere. The spatial distribution of the rhizosphere is a dynamic function of the soil matrix and plant properties, including root development and morphology, microbial colonization, nutrient uptake, root exudation and rhizodeposition (Dazzo and Gantner, 2012; Neumann and Romheld, 2002The correction is right.). Rhizodeposits include lost root cap and border cells, dead and lysed root cells, lost gasses, passively and actively released solutes (root exudates), and gelatinous material from the surface of roots (mucigel) (Curl and Truelove, 1986; Hinsinger et al., 2009; Jones et al., 2009).

Rhizodeposits play a crucial role for rhizosphere processes: stimulates microbial activity (Hinsinger et al., 2009; Kuzyakov and Domanski, 2000) and the production of enzymes (Asmar et al., 1994)

and, thus, nutrient availability and soil organic matter (SOM) decomposition (Cheng and Coleman, 1990). However, the higher enzyme activity of the rhizosphere than of root-free soil depends not only on microbial activity, but also on the direct release of enzymes by roots or by lysis of root cells (Jones et al., 2009; Marinari et al., 2014). On average, plants release 20% of photosynthesized carbon (C) through their roots in the form of high- and low-molecular weight organic compounds (Badri and Vivanco, 2009; Fischer et al., 2010; Hinsinger et al., 2006). The exact amount and quality of root exudates of an individual plant strongly depend on photosynthetic activity (Siczek and Lipiec, 2016) and root development (Aulakh et al., 2001), which are largely controlled by plant age. The temporal dynamics of enzyme activities in soil are affected by the quality and quantity of root exudates during each growth stage (Ren et al., 2016; Zhang et al., 2014).

While the interactions between plants and microorganisms are recognized as the major biotic factors influencing enzyme activities, abiotic factors such as temperature, water potential, pH, and soil texture are also important controls (Burns et al., 2013). Among these factors, temperature sensitivity of enzyme activity has recently re-

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ceived considerable interest because of its potential feedback to climate change (Davidson et al., 2006). Temperature sensitivity is commonly presented as the  $Q_{10}$  index, the factor by which reaction rate is multiplied when temperature increases by 10 °C (Birgander et al., 2013). When compared to respiration rates commonly assumed to have  $Q_{10}$  values of 2–3, enzyme activities are less temperature sensitive, with  $Q_{10}$  values < 2 (Browman and Tabatabai, 1978; Koch et al., 2007; Tabatabai, 1994). High temperatures generally increase enzyme activity (Nottingham et al., 2016; Razavi et al., 2017; Stone et al., 2012). However, the temperature sensitivity of enzymatic reactions also associates with variable factors, including temperature range, substrate supply, desiccation stress, etc. (Davidson et al., 2006). Higher temperatures commonly generate lower  $Q_{10}$  values (Razavi et al., 2016a; Tjoelker et al., 2001). Besides, when substrate concentration is low, a canceling effect (the absence or strong reduction of a response of an enzyme to temperature) decreases the expected enzymatic reaction rate (Berry and Raison, 1981; Davidson et al., 2006; Razavi et al., 2015). When  $Q_{10}$  of catalytic reactions is ~1, the reaction is restricted by temperature sensitivity of a bottle-neck process that accesses available substrate, e.g., during soil organic matter decomposition, by decomposition of recalcitrant or stabilized SOM (Ågren and Wetterstedt, 2007). When substrate diffusion varies with temperature, the temperature sensitivity of enzyme activity will be further affected (Davidson et al., 2006). Accordingly, the canceling effect can be an important phenomenon controlling the ‘actual’ temperature sensitivity of organic material decomposition in soils (Razavi et al., 2015; Von Lützow and Kögel-Knabner, 2009) and nutrient mobilization. Thus the strength of the canceling effect is affected by substrate availability and this can vary during plant growth. Despite theoretical predictions (Davidson et al., 2006), and experimental evidence (Blagodatskaya et al., 2016; Razavi et al., 2015), there is still a lack of data on the occurrence of canceling as dependent on temperature and substrate amount in the rhizosphere—the sphere with a high concentration of labile compounds.

*In situ* visualization of the spatio-temporal distribution of enzyme activity in critical spheres, such as the rhizosphere, and how it is affected by temperature is required to reveal complex interactions between microorganisms, enzymes, and SOM decomposition (Wallenstein and Weintraub, 2008). However, it is still an unsolved question whether and how temperature affects the dynamics and localization of enzymatic hotspots in the rhizosphere. The recently modified soil enzyme activity imaging technique—so called direct soil zymography (Razavi et al., 2016a,b; Sanaullah et al., 2016)—offers an opportunity to analyze the two-dimensional spatial distribution of enzymes in soil (Vandooren et al., 2013). Direct soil zymography enables the mapping of enzyme activity at the soil surface (Sanaullah et al., 2016), in biopores (Hoang et al., 2016a,b) the rhizosphere (Razavi et al., 2016a,b) and the detritosphere (Liu et al., 2017; Ma et al., 2017). Here for the first time, we quantitatively imaged the impact of temperature on the spatio-temporal distribution of enzyme activities in the rhizosphere during rice growth—one of the most important food crops in China. Our study aimed to illustrate: 1) how the spatial distribution of soil enzyme activities is affected by temperature and 2) how the impact of temperature varies with plant growth stage. We hypothesized that 1) high temperature, root development, and plant growth will increase enzymatic activity and hotspot area; 2) such an increase in hotspot area is enzyme dependent; and 3) there is no canceling effect in the rhizosphere of rice (*Oryza sativa* L.), which is an important agricultural crop for food production. Thus, we studied the spatio-temporal distribution of enzymes involved in P and N cycles and crucial for improving nutrient

availability. Phosphatase that catalyzes the degradation of phosphorous-containing organic compounds (Asmar et al., 1994; Eivazi and Tabatabai, 1988; Siber, 2000) and *N*-acetylglucosamine (e.g., chitinase), which accomplishes the decomposition of chitin to a low molecular weight chitooligomer (German et al., 2011; Hoang et al., 2016a), at two temperatures (18 and 25 °C) after 14 and 30 days.

## 2. Materials and methods

### 2.1. Sample preparation

Hydragric Anthrosol (Gong et al., 2007) developed from a granite parent material after very long, intensive subtropical weathering was collected from a rice field (113°19'52"E, 28°33'04"N, 80 m above the sea level) located at the Changsha Research Station for Agricultural and Environmental Monitoring, Hunan Province, China. Soil samples were collected from the Ap horizon (15% water content) and sieved (<4 mm) to remove coarse plant residues. The soil texture was 10.4% clay, 76.5% silt, and 13.1% sand. We grew 16 rice plants (*Oryza sativa* L. ‘Zhongzao 39’), each in a separate rhizobox with inner dimensions of 20.5 × 13.4 × 5.2 cm. The rhizoboxes were placed horizontally with one side open and then soil was slowly and continuously poured into the rhizoboxes through a 2 mm sieve to achieve uniform soil packing and to avoid soil layering. The open side was then closed, the samples were turned vertically, and they were gently shaken to achieve stable soil packing. The seeds were germinated on filter paper for 72 h. Then one seedling was planted in each rhizobox at a depth of 5 mm.

During 30 days of growth, the rhizoboxes were kept inclined at an angle of 45° so that the roots grew along the lower wall of the rhizoboxes. All samples were kept in climate-controlled chambers which were regulated by automatic temperature control system with a deviation of ±1 °C, set to 18 or 25 °C and a daily light period of 16 h with 300 μmol m<sup>-2</sup> s<sup>-1</sup> of photosynthetically active radiation intensity. The water level was maintained throughout the rice growing season at 2–3 cm above the soil surface to simulate the water condition in most paddy fields before zymography analysis.

### 2.2. Direct soil zymography

After cultivating rice for 14 and 30 days, direct soil zymography was applied as an *in situ* technique to study the spatial distribution of enzyme activity around the roots. We followed the protocol optimized by Razavi et al. (2016a,b). Visualization of enzyme activities involved using membranes saturated with 4-methylumbelliferone (MUF)-substrates, which become fluorescent when enzymatically hydrolyzed by a specific enzyme. 4-Methylumbelliferyl-*N*-Acetyl-α-D-glucosaminide (MUF-*N*-Ac) was used as substrate to detect *N*-acetyl-glucosaminidase (chitinase) activity; phosphatase activity was detected using 4-methylumbelliferyl-phosphate (MUF-Phos). Each of these substrates was separately dissolved to a concentration of 10 mM in MES buffer (Sigma-Aldrich, Germany). Polyamide membrane filters (Tao Yuan, China) with a diameter of 40 cm and a pore size of 0.45 μm and were cut into sizes adjusted for the rhizobox, and adjusted membranes were saturated with the substrates for each enzyme. The rhizoboxes were opened from the lower, rooted side and the saturated membranes were applied directly to the soil surface (Hoang et al., 2016b; Razavi et al., 2016b). After incubation for 1 h, the membranes were carefully lifted off the soil surface and any attached soil particles were gently removed using tweezers.

### 2.3. Image processing and analysis

Fluorescence visible on the zymograms under UV light shows the areas where substrate has been enzymatically hydrolyzed, and the intensity of fluorescence is proportional to the activity of the enzyme. To quantify this, we processed the zymograms in Matlab, according to Razavi et al. (2016b). Briefly, zymograms were transformed to 16-bit grayscale images as matrices and corrected for light variation and camera noise (Menon et al., 2007). We used the grayvalue obtained from the blank side of the samples as the reference point. After referencing the zymograms, we calculated an average background gray-value for the zymograms at the zero concentration point on the calibration lines and subtracted this value from all the zymograms. To quantify the zymogram images, a standard calibration for coloration the activities of various enzymes and the gray-value of zymogram fluorescence was prepared. The calibration function was obtained by zymography of 4 cm<sup>2</sup> membranes soaked in a solution of MUF with concentrations of 0.01, 0.2, 0.5, 1, 2, 4, 6, and 10 mM. The amount of MUF per area was calculated based on the solution volume taken up by the membrane and its size. The membranes used for calibration were imaged under UV light and analyzed in the same way that the samples were.

The resulting images were then used for further analysis. The roots were segmented as a threshold method in Matlab was used to detect the boundaries of the roots (Chaudhuri et al., 1989). The segmented roots and their lengths were calculated using the Euclidean distance map function in Matlab to calculate overall enzyme activity on the root surface.

Hotspots were distinguished from the surrounding area by the intensity of their color contrast in the digital images. Based on the image references and calibration lines, the color intensity of all pixels exceeding the average value (i.e., >0.7) were designated part of the enzyme activity hotspots (represented by a red color in the images). To confirm the boundaries, a one-way analysis of variance (ANOVA) was applied to assess the significant differences between independent variables (mean values of four adjacent pixels, i.e., equal to 0.1 mm). Significant results were then considered as the boundaries for each category (low and medium activities and hotspots) (Hoang et al., 2016b). Thus, ANOVA, followed by a Tukey's HSD test at a probability level of  $p < 0.05$ , confirmed the categories of enzyme activity. Homogeneity of variance and normality of the values were tested using a Levene's test and Shapiro Wilk's W test, respectively.

We used the conventional  $Q_{10}$  function (1) to examine variation in temperature sensitivity, and we expressed the temperature responses of each enzyme activity:

$$Q_{10} = \left( \frac{R(T_2)}{R(T_1)} \right)^{\frac{10}{(T_2 - T_1)}} \quad (1)$$

where  $R(T_1)$  and  $R(T_2)$  are the rates ( $R$ ) of a process or reaction at one of two temperatures ( $T_1$  or  $T_2$ ) (Karhu et al., 2010; Kirschbaum, 1994). Reaction rate at a distance of 0.5 mm at each temperature were used for the calculation of temperature sensitivity of both enzymes.

### 3. Results

#### 3.1. Response of enzyme activity and hotspot area to temperature and rice growth

Higher temperatures resulted in greater activities of both chitinase (Fig. 1) and phosphatase (Fig. 2). At 25 °C, average activity was greater for chitinase and phosphatase on day 14 (Figs. 3 and 4). Total enzyme activities declined by 30 days for both enzymes. However, the decrease in phosphatase was slight and was accompanied by an increase in hotspot area (Fig. 4). The total hotspot area of enzyme activity was slightly higher with higher temperature but was highly affected by rice development. Fourteen days after planting, chitinase activity was not associated with the roots and was distributed throughout the soil at both temperatures. The hotspots were also dispersed throughout bulk and rhizosphere soil (Fig. 3) and accounted for 2% and 2.5% of total soil surface area at 18 °C and 25 °C, respectively. Over time, the hotspot area of chitinase decreased with a shift in localization pattern from dispersed to mainly associated with roots throughout the whole rhizobox (Fig. 3). However, the hotspots contributed to 2% of the total phosphatase activity area after 14 days of rice growth and increased considerably to 6% and 7% at 18 °C and 25 °C, respectively, during this period (Fig. 4). The localization of phosphatase hotspots followed a similar pattern as chitinase. Thus, warmer temperatures were related to greater total enzyme activity; however, time affected the localization of hotspots for both tested enzymes.

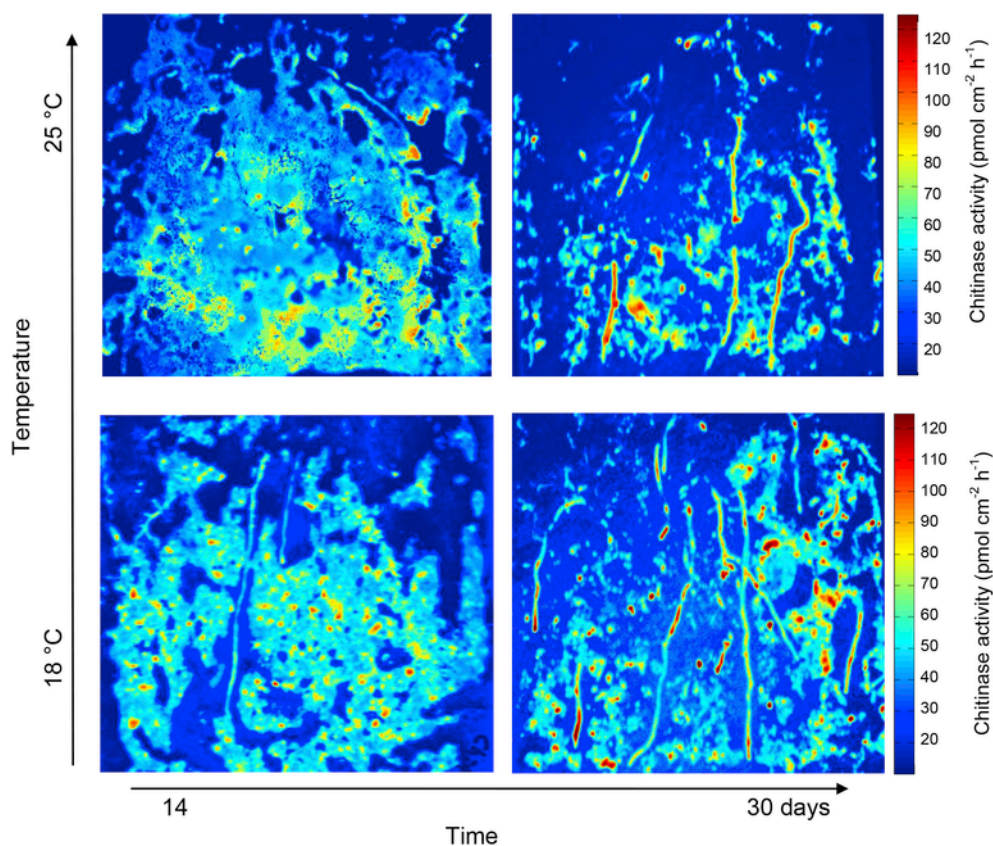
Enzyme activity as a function of distance from root center to the soil revealed that the extent of the rhizosphere is temporally constant (Fig. 5). However, enzyme activity increased in the rhizosphere over time (Fig. 5), and the rhizosphere extent was enzyme specific. For instance, the distribution of phosphatase activity was broader from the root surface (2.5–3.5 mm) compared with that of chitinase (1–1.5 mm).

#### 3.2. Canceling effect

In response to warming, phosphatase and chitinase activities increased in the rhizosphere (Fig. 5). However, the temperature sensitivity of phosphatase and chitinase decreased with increasing distance from the root surface corresponding to  $Q_{10}$  values of 1.4 and 1.0, respectively (Fig. 6). Thus, the canceling effect was pronounced at some distance away from root surface and it was enzyme-specific: 2.5–3.5 mm for phosphatase and 1.0–1.5 mm for chitinase (Fig. 6).

### 4. Discussion

In line with our first hypothesis (H1), the overall enzyme activities in soil were greater at higher temperatures, and the percent hotspot area at 25 °C was greater than it was at 18 °C (Figs. 3 and 4). Such increments were likely due to increased microbial activity (Bradford et al., 2008; Steinweg et al., 2008), and enzymatic activities (Kirschbaum, 2006) increase root exudates at warm temperatures. However, remarkably, hotspot localization (not activity) of both chitinase and phosphatase were temperature independent (Figs. 1 and 2). After 14 days, the hotspots of both the N and P cycle enzymes were spread more or less evenly throughout the rhizobox, while after 30 days they were clustered near the roots. Thus, heterogeneity of enzyme hotspots increased with time based on the constant location of the enzyme source—here, the roots.



**Fig. 1.** Spatial distribution of chitinase activity at 18 and 25 °C after 14 and 30 days of rice growth. Side color maps indicate enzyme activities ( $\text{pmol cm}^{-2} \text{h}^{-1}$ ).

Such a pattern is primarily attributed to inputs of easily degradable organic compounds from the roots and the resulting stimulation of rhizosphere microorganisms (Kuzakov and Domanski, 2000) and direct release of enzymes by roots (Asmar et al., 1994). Furthermore, the hotspots are relevant not only in terms of organic matter availability and C limitation, but also in terms of other specific factors limiting microbial activity or process rates, including soil moisture and oxygen availability. Hotspot localization is not confined to labile C inputs but is also controlled by the removal of any factors limiting microbial processes (Kuzakov and Blagodatskaya, 2015). In the rice rhizosphere, this limitation is access to oxygen (Larsen et al., 2015). Thus, the water saturation of paddy soil likely weakens the temperature effect on formation of enzymatic hotspots.

Similar hotspot areas observed in the rhizosphere and in bulk soil at an early stage of growth (day 14) could be due to the roots being too small to affect microbial activities in soil. With rice growth, chitinase activity in the rhizosphere and bulk soil decreased, but much lower activity in bulk soil at day 30 indicated a decline in exudation of N containing organic matters. In contrast, phosphatase activity increased in the rhizosphere during rice growth. Such an increase is mainly connected to the production of this common enzyme by both plants and microorganisms (Blagodatskaya and Kuzakov, 2008; Dick and Tabatabai, 1984; Nannipieri et al., 2011). Furthermore, rice root-derived organic substrates shift from organic acids toward sugars with advancing plant age (Aulakh et al., 2001) and thus provide different energy sources for microbial metabolisms (e.g. carbon compounds) during an entire growing season. Therefore, we assume that variation in availability of C compounds controls phosphatase production and activity. Thus, in line with our hypothesis (H2), the spatio-temporal dynamics of enzyme activities are enzyme-specific and

strongly related to plant growth (Fig. 7), which regulates root exudation.

Enzyme activity as a function of distance from the root demonstrates that the rhizosphere extent is temporally constant (Fig. 5). Hence, while enzyme activities increased with plant growth, the radial distribution of the rhizosphere remained constant. The spatial stability of the rhizosphere extent reflects the equilibrium between the input—release from roots and diffusion—with the output—microbial decomposition and other inactivation of the enzymes. This stable pattern is an excellent strategy for plants to acquire nutrients in a highly efficient manner in a narrow root zone independent of root age (Grossmann et al., 2011).

In agreement with our third hypothesis (H3), enzyme activities stepwise decreased with distance from the root surface due to a reduction of substrate availability and enzyme production. Correspondingly, the  $Q_{10}$  values for reaction rates were always  $>1$  at the root-soil interface (average range: 1.3–1.4). Independent of enzymes, canceling was never observed in the vicinity of the roots (Fig. 6). Thus, the canceling effect is a substrate concentration-dependent phenomenon.

To our knowledge, this is the first study exploring the canceling effect in the rhizosphere. Absence of canceling at the root-soil interface for phosphatase and chitinase revealed that warming will accelerate P and N mobilization in the rhizosphere. Such a general reduction in temperature sensitivity ( $Q_{10}$ ) confirms theoretical predictions (Davidson et al., 2006) and experimental observations at low substrate concentrations (Razavi et al., 2015; Blagodatskaya et al., 2016).

The occurrence of a canceling effect for phosphatase and chitinase a few millimeters away from the root surface suggested slower decomposition of P and N containing organic compounds. Therefore, canceling can be considered a natural mechanism that can reduce the



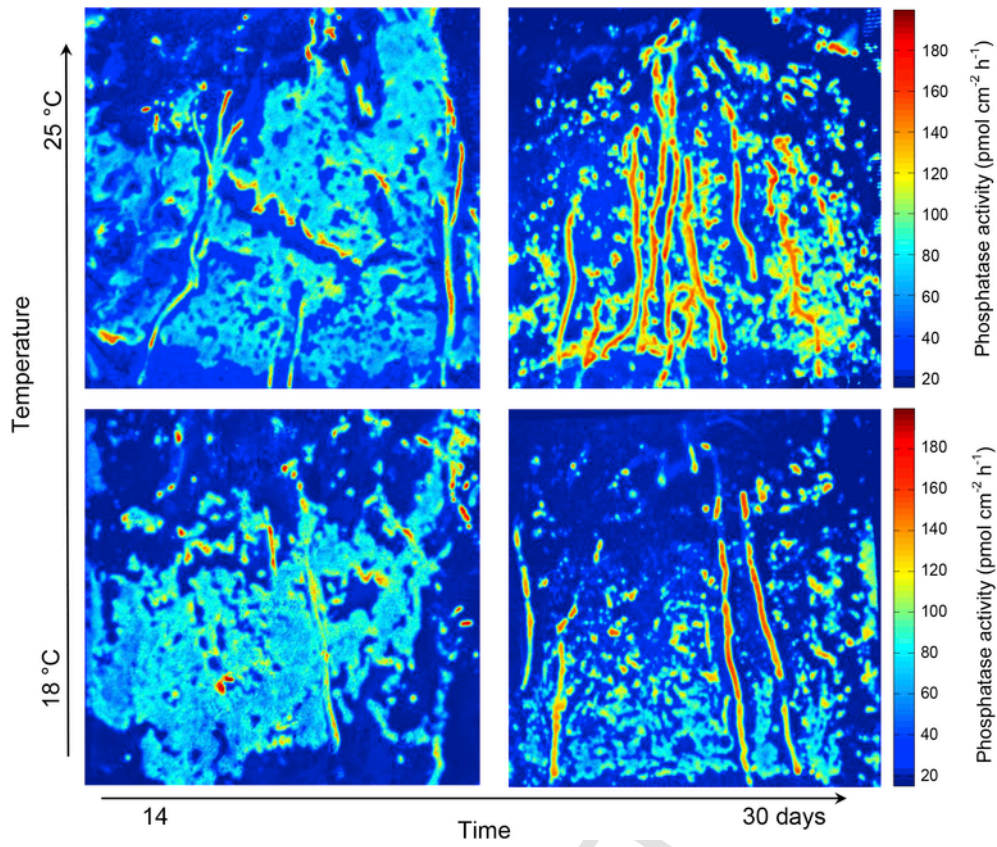


Fig. 2. Spatial distribution of phosphatase activity at 18 and 25 °C after 14 and 30 days of rice growth. Side color maps indicate enzyme activities ( $\text{pmol cm}^{-2} \text{h}^{-1}$ ).

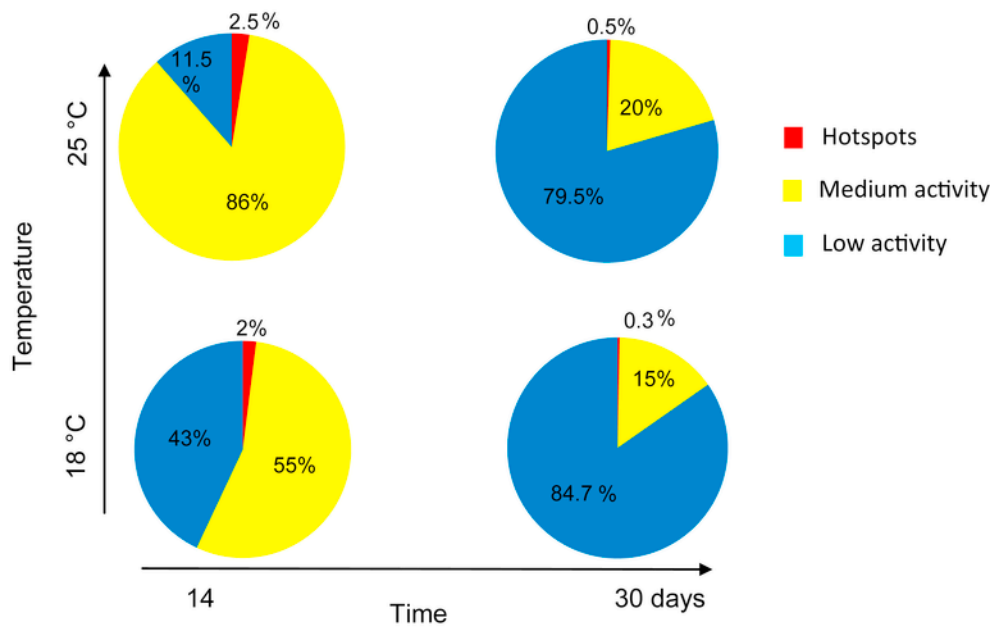
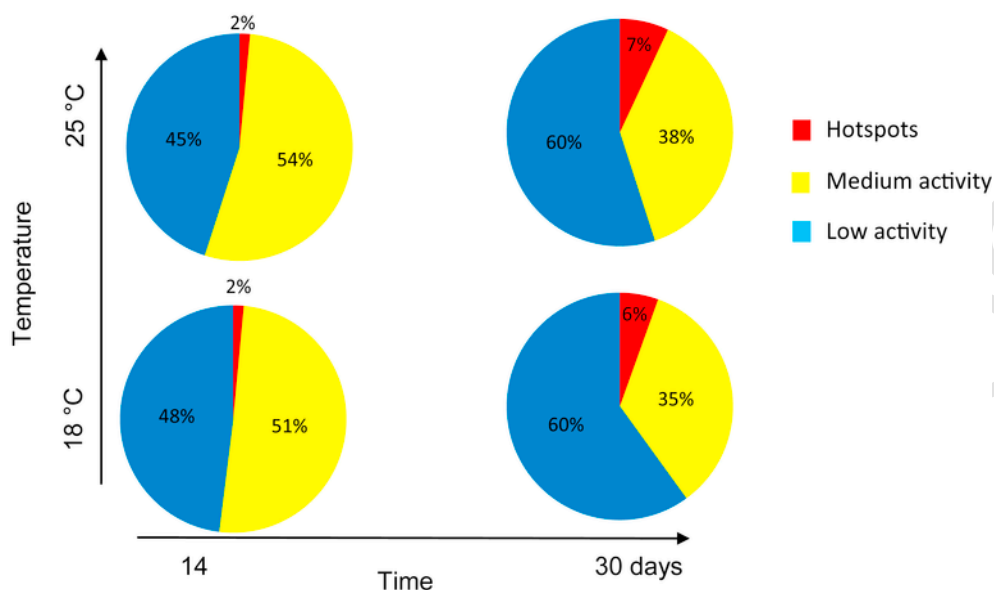
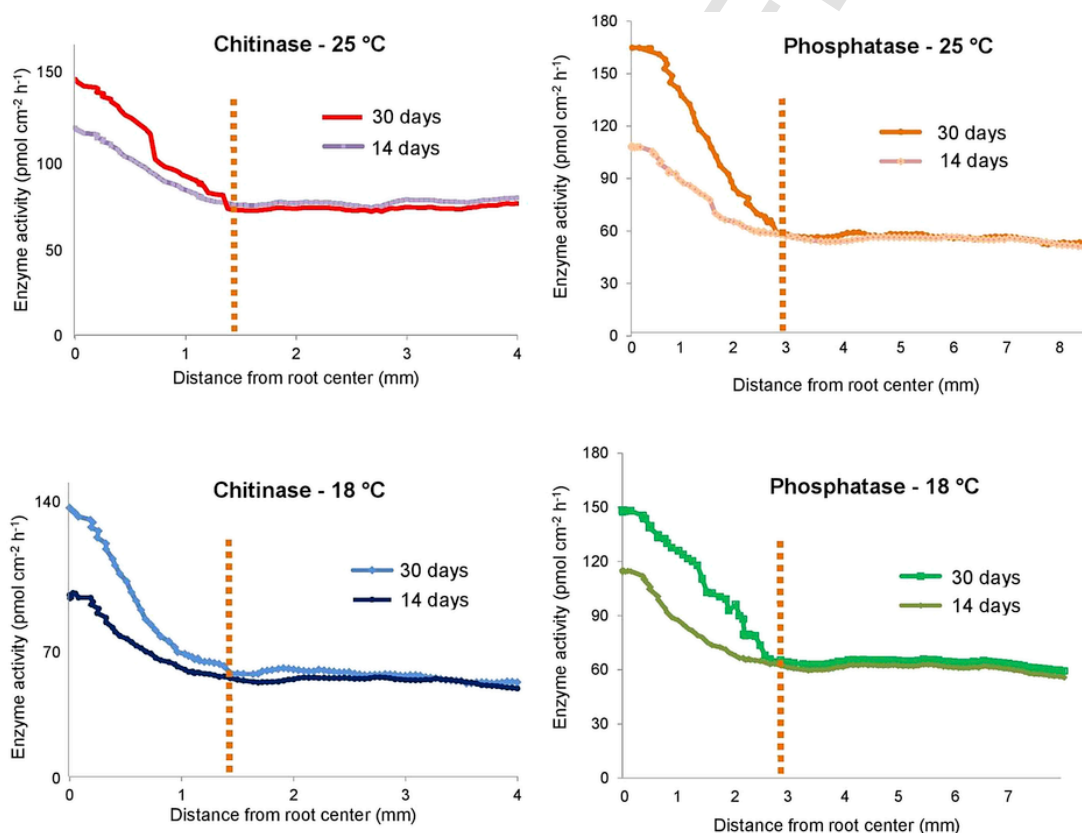


Fig. 3. Contribution of three classes of chitinase activity to total activity on the zymogram area. Data represent mean values of three independent rhizotrons ( $n = 3$ ) each of them with 3 roots. Blue: low activity; yellow: medium activity, red: hotspots. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



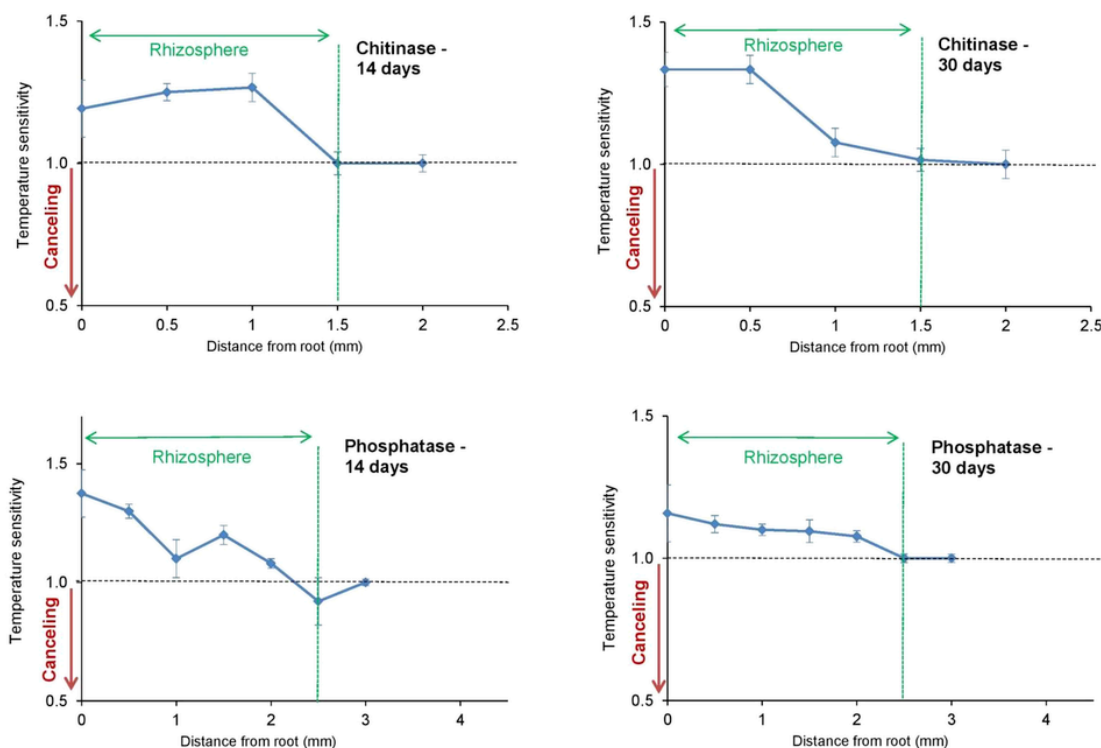
**Fig. 4.** Contribution of three classes of phosphatase activity to total zymogram area considering the effect of the number of roots. Data represent mean values of three independent rhizotrons (n = 3). Blue: low activity; yellow: medium activity, red: hotspots. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



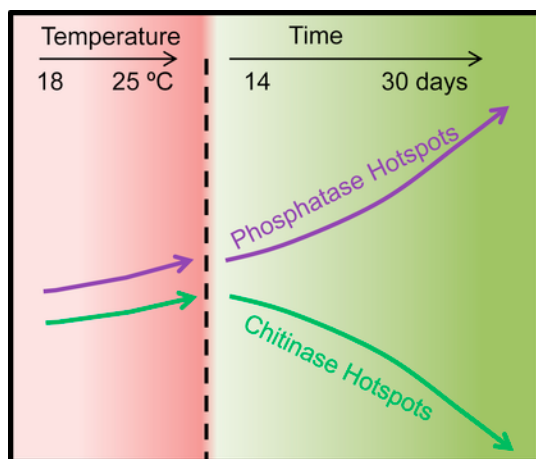
**Fig. 5.** Enzyme activity as a function of distance from root center for rice (*Oryza sativa*). Rhizosphere extent remained constant, independent of time and temperature, but was enzyme specific: 1.5 mm for chitinase and 3 mm for phosphatase. Temperature and time increased chitinase and phosphatase activity.

consequences of global warming on the microbial decomposition of soil organics at moderate temperatures. Canceling resulting from microbial and abiotic interactions may be responsible for weaker nutrient mobilization in root-free soil compared to the rhizosphere. Under

high substrate concentration in the vicinity of the root, however, the regulation of hydrolytic activity by canceling in response to warming is of minor relevance given that canceling was never observed near the root (Fig. 6).



**Fig. 6.** Absence of canceling effect in the rhizosphere and presence of canceling in the bulk soil. Canceling effect is shown at a distance of a) 1 mm for chitinase and b) 2.5 mm for phosphatase.



**Fig. 7.** General distribution pattern of enzyme activity hotspots in paddy soil. Warming from 18 to 25 °C only marginally affected hotspot distribution, but the redistribution of hotspots depended very strongly on time.

Overall, for the first time we showed that the extent of enzyme activity in the rhizosphere is temporally constant. Thus, despite well-known high dynamics of the rhizosphere, the spatial pattern of enzyme activity around the roots remains stable. The results of this study indicated that high temperature had a positive impact on total enzyme activities, but contrary to our hypothesis, it did not considerably affect the spatial distributions of hotspots. Instead, rice growth and root development impacted the percent of enzymatic hotspot area and localization patterns strongly. Additionally, occurrence of canceling in root-free soil and its absence at the root-soil interface, revealed that warmer temperatures will accelerate nutrient mobilization in the

rhizosphere more than in the root-free soil. Finally, we conclude that the constant extent of the rice rhizosphere under temperature control revealed that in paddy soil warming did not affect rhizosphere size and the area where enzymatic mobilization of nutrients occur. This should be considered in modeling of rhizosphere dynamics and the corresponding effects on soil properties and functions in a warmer world.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.soilbio.2017.06.005>.

## Uncited references

Bodelier, 2011, Choi et al., 2009, Lu et al., 2004, Sauer et al., 2006, Song et al., 2012, Yuan et al., 2015, Bekku et al., 1997, Zhao et al., 2016 please delete this reference..



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